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EXPRESSION OF CHIMERIC RECEPTOR COMPOSED OF IMMUNOGLOBULIN-DERIVED V REGIONS AND T-CELL RECEPTOR-DERIVED C REGIONS

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SUMMARY : Chimeric genes composed of immunoglobulin (Ig)-derived variable (V) regions and T-cell receptor (TCR)-derived constant (C) regions were constructed. The V_L and V_H genes showing anti-phosphorylcholine(PC) activity were used in this study. Two pairs of chimeric genes, V_L-C_β and V_H-C_α genes, and V_L-C_α and V_H-C_β genes, were inserted into an expression vector containing both Ecogpt and neo genes, and transfected into EL4 cells. Cells which express both chimeric receptor molecules were established. The activity of the transformants to the antigen was examined by using stopped-flow fluorometry. An increase in the concentration of cytoplasmic calcium ion was observed after addition of Staphylococcus pneumoniae R36A bacteria grown in the choline-containing medium which express PC molecules, but not after the PC-negative bacteria grown in the ethanolamine-containing medium. © 1987 Academic Press, Inc.

TCR is a heterodimer of disulfide-linked α and β chains (1). The essential features of both chains are rather similar to those of Ig(2). N-terminal regions of both chains are variable and form a dimeric antigen-combining site (3). In contrast to the recognition of antigen by antibody, where the antibody can bind to free antigen, the TCR does not recognize antigen alone but only in association with MHC molecules (4). To determine whether the difference in antigen recognition between T and B

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Abbreviations : Ig, Immunoglobulin; TCR, T-cell receptor; V, Variable; C; constant; H, heavy; L, light; PC, phosphorylcholine; ECO-MOL, ecotropic Moloney virus; LTR, long terminal repeat.

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cells derives from the structural difference in V regions of TCR and Ig, we constructed chimeric genes composed of Ig-derived V regions and TCR-derived C regions. The V_L and V_H gene showing anti-PC activity were used in this study. The V genes of the light (L) and heavy (H) chains of anti-PC myelomas and hybridomas have been well characterized (5,6). Only one germline V_H gene (5) and three germline V_L genes (6) are responsible for the anti-PC specificity. Cells which express chimeric receptor molecules were established. It is known that the activation of T lymphocytes is accompanied by an increase in the concentration of cytosolic calcium (7). The activity of the transformants to the antigen was examined by using stopped-flow fluorometry (8). The chimeric receptor molecules expressed on the transformants had the capacity to react with PC antigen and trigger T cell activation.

MATERIALS AND METHODS

Active V_H gene was isolated from myeloma TEPC15 by using J_H gene-containing DNA fragment as a probe (5). Active V_L gene isolated from myeloma S107 was donated by M.D. Scharff (Albert Einstein College) (9). The C genes of T-cell receptor α and β chains were isolated from C3H and C57BL/6 mouse DNA by using cDNAs of α and β chains (2,10) donated by M.Davis (Stanford University) as probes, respectively. The ecotropic Moloney virus (ECO-MOL) long terminal repeat (LTR) fragment was donated by M.Ishimoto (Kyoto University) (11). Plasmid pAGE145 was described previously (12). The bacteria and the eukaryotic cells containing this plasmid show kanamycin resistance at a concentration of 10 γ/ml and G418 resistance at a concentration of 1 mg/ml, respectively. The nucleotide sequence of the constructed plasmids was determined by the M13-dideoxy method (13). EL4 cell was obtained from T.Taniguchi (Osaka University). Transfection experiments were done by protoplast fusion as described (14). Total RNA was extracted from cells by the Guanidinium-CsCl method (15). Poly A-containing RNA was purified with the oligo (dT) cellulose column (15). Northern hybridization was performed as described (16). Anti-V_H antibody (17) and anti-T15 idiotypic antibody (18) were donated by D.Givol (Israel) and C.Heusser (Basel), respectively. Stopped-flow fluorometry was carried out as follows. Fura 2-loaded T-cells (19) (final 10^6 cells/ml) were mixed with the target bacteria (final 4 X 10^8 cells/ml) at 25°C. Stopped-flow fluorescence was measured with a Union Giken stopped-flow spectro-photometer RA401 in combination with a microcomputer RA-450 system (8). Excitation wavelength : 335nm. We used a Hoya Y46 cut filter (which allows the emitted light with wavelength longer than 460 nm to come into the detector) in the fluorescence measurements.

RESULTS

Figure 1 shows the construction procedures for four kinds of chimeric genes: V_L-C_β, V_H-C_α, V_L-C_α and V_H-C_β. To express the chimeric genes in T cells, we used the ECO-MOL LTR as a transcriptional promoter. Since translation begins at the 5'-proximal AUG triplet in eukaryotic mRNAs (20), we determined the nucleotide sequence between the promoter and

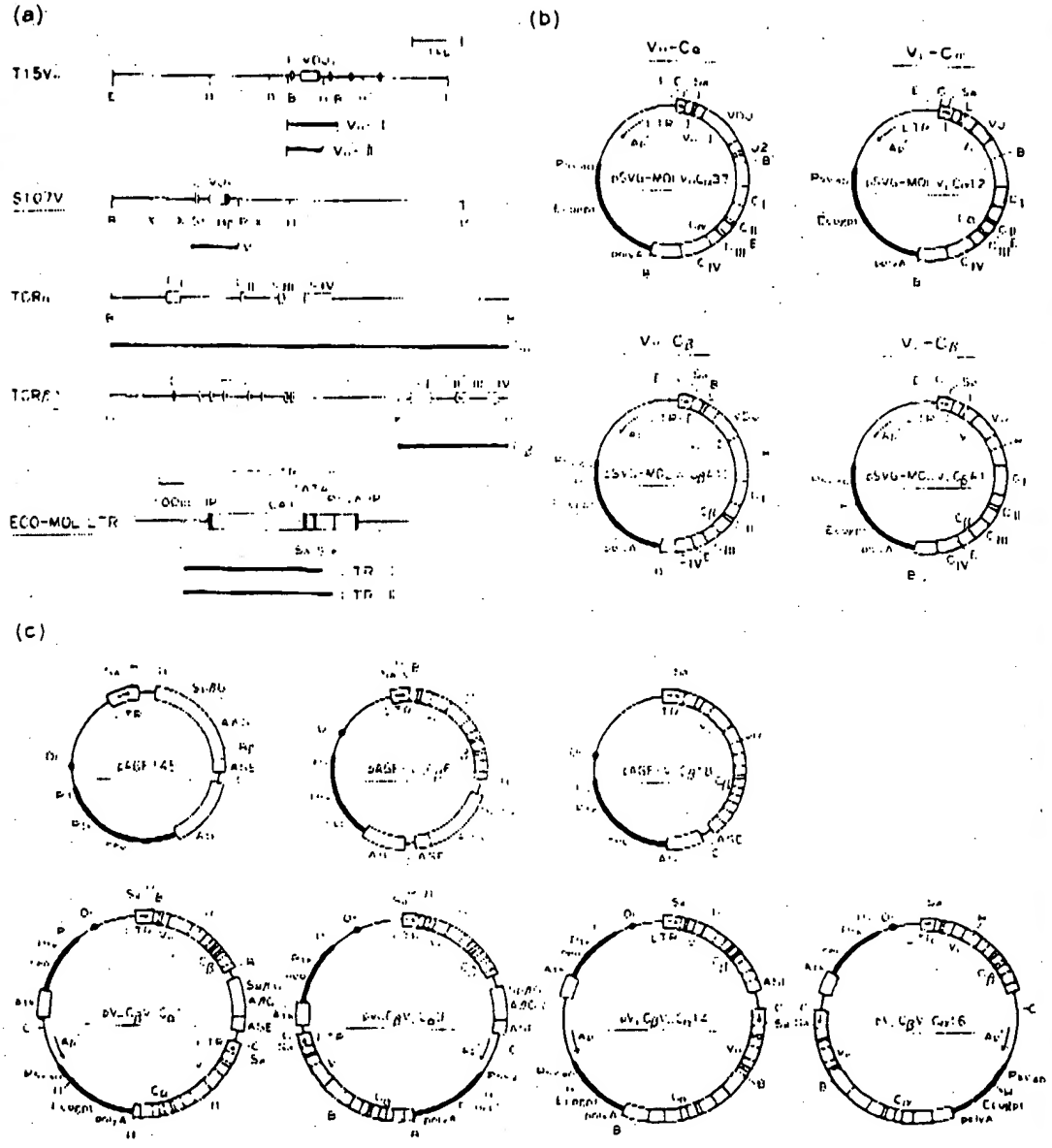

Fig.1. Construction of the chimeric genes. (a)Restriction maps of the clones. The DNA fragments used for the construction of plasmids are indicated by thick lines. Restriction Enzymes: E,EcoRI; B,BamHI; H,HindIII; X,XbaI; St,StuI; Bg,BglII; P,PstI; K,KpnI; C,ClaI; Sa,SacI; S,SmaI. (b)For the construction of pSVG-MOLV$_H$C$_\alpha$37, the ClaI-SmaI fragment of the ECO-MOLLTR(LTR-I) was connected to the BamHI-BamHI fragment of T15V$_H$ (V$_H$-I), after the BamHI site at the 5' upstream of V$_H$ gene was changed to a flush end with Klenow enzyme. The resulting ClaI-BamHI fragment was connected to the BamHI-BamHI fragment of TCR α chain as in the same polarity, and inserted into plasmid pSV2gpt. The EcoRI-ClaI region at the upstream of the LTR sequence was derived from pBR322 DNA. ; For the construction of pSVG-MOLV$_L$C$_\alpha$12, the LTR-I fragment was connected to the StuI-BglII fragment of S107V$_L$(V$_L$). The resulting fragment was connected to the BamHI fragment of C$_\alpha$ gene after changing the BglII site into BamHI site by using a BamHI linker, and inserted into plasmid pSV2gpt. ; For the construction of pSVG-MOLV$_H$C$_\beta$A11, the ClaI-KpnI fragment of the ECO-MOL

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the leader of V_H and V_L genes in the constructed plasmids. There were no ATG sequences in the 5' upstream regions (Fig.2). In an initial trial for transformation experiments, the EL4 cells, presumed to be a helper T cell(21), were transfected with two plasmid DNAs: pSVG-MOLV$_H$C$_\alpha$37 and pSVG-MOLV$_L$C$_\beta$(neo), or pSVG-MOLV$_L$C$_\alpha$12 and pSVG-MOLV$_H$C$_\beta$(neo), simultaneously, by protoplast fusion (14) and selected in the gpt (22) and neo selection medium (23). However, we did not obtain any transformants showing both gpt and neo resistance. We constructed the plasmids : pV$_H$C$_\beta$V$_L$C$_\beta$1, pV$_H$C$_\beta$V$_L$C$_\alpha$3 pV$_L$C$_\beta$V$_H$C$_\alpha$14 and pV$_L$C$_\beta$V$_H$C$_\alpha$16, containing two chimeric genes in each plasmid DNA (Fig.1c). From 4 x 10^6 EL4 cells, in the gpt selection medium, we obtained 7, 22, 13 and 6 transformants transfected with pV$_H$C$_\beta$V$_L$C$_\alpha$1, pV$_H$C$_\beta$V$_L$C$_\alpha$3, pV$_L$C$_\beta$V$_H$C$_\alpha$14 and pV$_L$C$_\beta$V$_H$C$_\alpha$16, respectively. In the neo selection medium, we obtained 2, 10, 9 and 3 transformants transfected with the above plasmid DNAs, respectively. By Northern hybridization (16) with the V_H and V_L probes, we searched for cells expressing both chimeric genes, and obtained two transformants : EL4-5 containing pV$_H$C$_\beta$V$_L$C$_\alpha$3 and EL4-14 containing pV$_L$C$_\beta$V$_H$C$_\alpha$14, as neo resistant cells. EL4-5 gave a band at 1.35 kb with the V_H probe and at 1.45 kb with the V_L probe(Fig.3). EL4-14 gave a band at 1.45 kb with

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LTR(LTR-II) was connected to the BamHI-HindIII fragment of TIsV$_H$(V$_H$-II) after changing the KpnI and the BamHI sites into flush ends with Klenow enzyme, which creates a BamHI site at the junction point. The resulting fragment was connected to the KpnI-HindIII fragment of C_β gene after changing the KpnI and HindIII sites into HindIII and BamHI sites, respectively, by using linkers, and inserted into plasmid pSV2gpt. ; For the construction of pSVG-MOLV$_L$C$_\beta$A1, the protocol was the same as that of pSVG-MOLV$_L$C$_\alpha$12 except for the change of the BglII site into HindIII site instead of BamHI site. Independently, we constructed four other plasmids whose structures are essentially the same as shown in (b) except for neo resistance gene in place of Ecogpt gene by using plasmid pSV2neo instead of pSV2gpt. We named them pSVG-MOLV$_H$C$_\alpha$(neo), pSVG-MOLV$_L$C$_\alpha$ (neo), pSVG-MOLV$_H$C$_\beta$ (neo) and pSVG-MOLV$_L$C$_\beta$ (neo), respectively. (c)For the construction of the plasmids containing two chimeric genes in one plasmid DNA, pAGE145(31) was used as a vector. The LTR is the ECO-MOLLTR and a polylinker shown in Fig.2 was inserted into the SmaI site. The designations are as follows: SpβG, AβG, ASE and Ark are β-globin splicing signal, β-globin polyA signal, SV40 early gene polyA signal and thymidine kinase polyA signal, respectively. P1 and Prk are the promoters of P1 and thymidine kinase genes. The BamHI-BamHI fragment of pSVG-MOLV$_H$C$_\beta$A11 containing the chimeric V_H-C_β gene was inserted into the BamHI site of pAGE145, resulting in the plasmid pAGE-V$_H$C$_\beta$6. The ClaI-digested pSVG-MOLV$_L$C$_\alpha$12 was connected to the ClaI-digested PAGE-V$_H$C$_\beta$6, resulting in two clones: pV$_H$C$_\beta$V$_L$C$_\alpha$1 and PV$_H$C$_\beta$V$_L$C$_\alpha$3. The SacI-BglII fragment of pAGE145 was replaced by the SacI-BamHI fragment of pSVG-MOLV$_L$C$_\beta$A1 containing the chimeric V_L-C_β gene, resulting in plasmid pAGE-V$_L$C$_\beta$18. The ClaI-digested pSVG-MOLV$_H$C$_\alpha$37 was connected to the ClaI-digested pAGEV$_L$C$_\beta$18, resulting in two plasmids: pV$_L$C$_\beta$V$_H$C$_\alpha$14 and pV$_L$C$_\beta$V$_H$C$_\alpha$16. The sizes of the four plasmids: pV$_H$C$_\beta$V$_L$C$_\alpha$1, pV$_H$C$_\beta$V$_L$C$_\alpha$3, pV$_L$C$_\beta$V$_H$C$_\alpha$14, and pV$_L$C$_\beta$V$_H$C$_\alpha$16 are 23, 23, 21.6 and 21.6 kb, respectively. The overall structures are confirmed by restriction mapping, Southern hybridization and nucleotide sequencing of the connected regions. The sizes of each plasmid and gene shown in (b)(c) and (d) are arbitrary.

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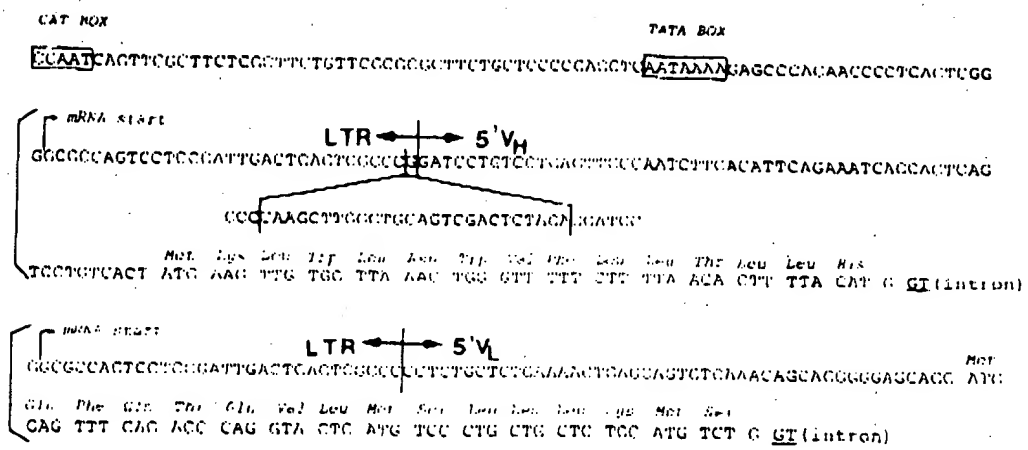

Fig.2. Nucleotide sequence from the promoter region to the first intron of the constructed V_H and V_L genes. The nucleotide sequence of the promoter was as described(11). The junction points of the LTR and V genes are at the SmaI or KpnI site in the ECO-MOI LTR, the BamHI site in the 5' upstream of T15 V_H gene and the StuI site in the 5' upstream of S107 V_L gene as described in the legend of Fig.1. Since a polylinker was inserted into the SmaI site in plasmid pAGE145 (12), there are 26 extra nucleotides at the boundary of the LTR and 5' V_H in pAGE-$V_H C_\gamma$ 6. The determined sequences are the same as the published sequence(5) except for a two nucleotide deletion in the 5' untranslated region of the V_H gene. The position of the initiation codon in S107 V_L gene is estimated from the locations of the octamer ATTTGCAT(33) and TATA-like sequences whose portions were removed from the constructed plasmids.

the V_H probe and at 1.25 kb with the V_L probe. The size of each band indicates that RNA splicing occurred between an Ig-derived J gene and a TCR-derived C gene. The size differences should reflect those of the 3' end structure of the constructed genes(Fig.1).

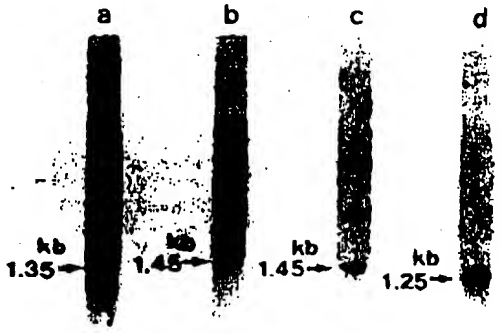

Fig.3. Northern hybridization of the transformants with the V_H and V_L probe. PolyA-containing RNA was prepared from each transformant. Two micrograms of RNA were subjected to 1% agarose gel electrophoresis. The size was estimated using 28S and 18S rRNA as size markers. Hybridization was carried out with (a)(c) the V_H probe (which corresponds to V_HII in Fig.1a), and (b)(d) the V_L probe (which corresponds to V_L in Fig.1a). (a)(b) EL4-5, (c)(d) EL4 14.

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To determine whether translation of mRNAs and expression of the chimeric receptor molecules on the cell surface occurred in these cells, we used two monoclonal antibodies: anti-V_H antibody (17) and anti-T15 idiotypic antibody (18). However, the EL4 used as a recipient cell itself reacted with anti-T15 idiotypic antibody, presumably due to the crossreactivity to Thy-1 which shares the same epitope as T15 idiotype (24), or some other surface molecules, and the results with anti-V_H antibody were ambiguous because of the high background. Because of these problems, we directly examined the activity of the transformants to the antigen without identification of the proteins.

It is known that the activation of T lymphocytes is accompained by an increase in the concentration of cytosolic calcium (7). After binding to target cells, T lymphocytes first increase their membrane fluidity and, then, calcium is released from intracellular stores (8). After that, calcium influx occurs from the external medium into T lymphocytes (8). This increase in the concentration of cytoplasmic Ca^{2+} ion can be traced by using stopped-flow fluorometry (8) with a fluorescent probe. As the source of PC antigen, we used heat-killed Staphylococcus pneumoniae R36A bacteria (25) grown in the choline-containing medium which express PC molecules,[9] and as the negative control, we used heat-killed PC-negative bacteria grown in ethanolamine-containing medium (27). Figure 4 shows that in both EL4-5 and EL4-14 cells, calcium influx was observed after mixing the T cells with PC-positive bacteria but not with PC-negative bacteria, and that EL4 itself did not react with either PC-positive or PC-negative bacteria. These results indicate that the chimeric receptor molecules presumably expressed in the transformants have the capacity to react with PC antigen and trigger T cell activation.

DISCUSSION

The TCR is associated with the T3 complex(28). Furthermore, the Lyt2/T8 and L3T4/T4 surface glycoproteins are thought to be involved in the interactions between T cells and their target cells (29). However, two groups (30,31) have presented direct evidence that the α and β chains of TCR alone may define the dual, namely antigen and MHC, specificity. Transfection and expression of the genes for a particular α and β pair allow the recipient cell to respond to the combination of antigen and allelic MHC product recognized by the donor T cell (30,31). The difference in antigen recognition between B and T cells may originate in the structural difference in Ig V regions and TCR V regions, although computer analyses (32), based on published sequence data, indicate that the

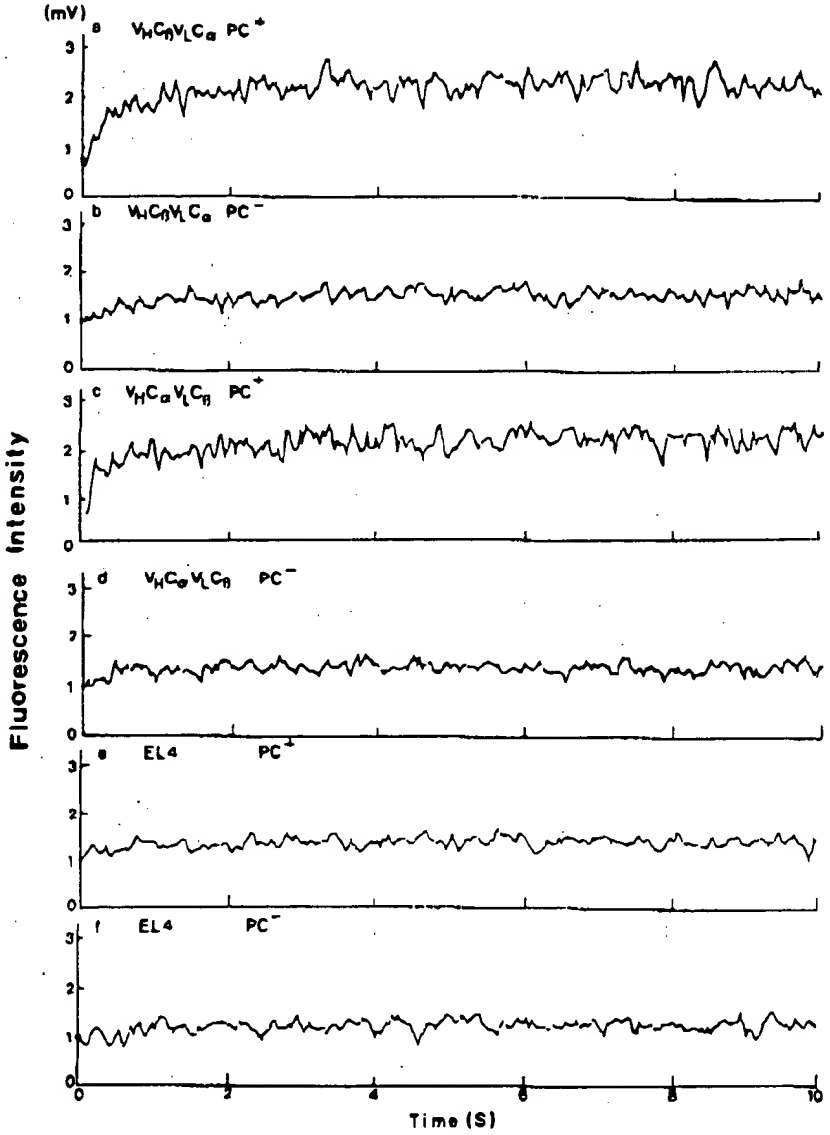

Fig.4. Stopped-flow fluorometry traces indicating the effects of the antigens on the T-cells with chimeric receptors.
The data are shown as the average of 15 times measurements.
(a) Effect of PC^+ on the fluorescence of fura 2-loaded EL4-5.
(b) Effect of PC^- on the fluorescence of fura 2-loaded EL4-5.
(c) Effect of PC^+ on the fluorescence of fura 2-loaded EL4-14.
(d) Effect of PC^- on the fluorescence of fura 2-loaded EL4-14.
(e) Effect of PC^+ on the fluorescence of fura 2-loaded EL4.
(f) Effect of PC^- on the fluorescence of fura 2-loaded EL4.

α and β chains of TCR are organized into immunoglobulin-like domains consisting of multistranded antiparallel β-sheet bilayers. Our present experiments indicate that the chimeric receptor composed of Ig-derived V regions and TCR-derived C regions has the capability to trigger T cell

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activation with the antigen alone, without MHC molecules. Since calcium influx is an early transmembrane event (8), we are now examining whether helper and cytolytic functions occur with antigens. If this is the case, the ability of Ig to bind to free antigens could be transferred into T cells. In future, it might become possible for T cells recognizing any antigens without MHC restriction to be produced by the technique described in this paper.

Recently, Gascoigne et al.(33) reported the expression of reverse chimeric genes composed of TCR-derived V regions and Ig-derived C regions. Although a chimeric protein V_α-$C_{\gamma 2b}$ combined with a normal λ light chain to form an apparently normal tetrameric immunoglobulin molecule that was secreted, it did not combine with a chimeric V_β-C_κ chain (33). The reason for formation of our chimeric chains : V_H-C_β and V_L-C_α, and V_H-C_α and V_L-C_β, although not directly demonstrated, might be related to the Ig's character in that V_H and V_L domains can be intrinsically associated without C domains to form an F_V fragment (34).

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